Igfbp3 Modulates Cell Proliferation in the Hair Follicle

To the Editor:

IGF-I has mitogenic functions but also plays a role in differentiation (Stewart and Rotwein, 1996; Musaro and Rosenthal, 1999; Hsieh *et al*, 2004). In skin, transgenic *Igf-I* expression causes epidermal hyperproliferation and stimulation of vibrissa growth, respectively (Bol *et al*, 1997; Su *et al*, 1999). In the interplay with IGF-I, IGF-binding proteins act either as agonists or antagonists depending on experimental conditions (Clemmons, 1992). Furthermore, they appear to have IGF-independent (e.g., pro-apoptotic) functions (Rajah *et al*, 1997; Hong *et al*, 2002). In previous reports, *Igfbp3* has been demonstrated to be expressed by the dermal papilla (Batch *et al*, 1996). In mouse, expression dramatically increases with the onset of catagen, suggesting a possible role in the control and/or execution of follicle regression (Schlake *et al*, 2004). Reports about transgenic *Igfbp3* expression in mouse using ubiquitous promoters offered no information as to the protein's role in hair follicles (Murphy *et al*, 1995; Modric *et al*, 2001).

To address this function, we sought to enhance *Igfbp3* expression during anagen (all experiments were approved by the MPI of Immunobiology). For this transgenic approach, we chose the involucrin promoter that drives gene expression in differentiating keratinocytes of the inner root sheath and of the medulla of hair follicles (Carroll *et al*, 1993). All transgenic mouse lines show strong transgene expression in the hair follicle (Fig 1*A* and *B*). Wild-type and ivl::Igfbp3 transgenic offsprings could be distinguished from each other by morphological criteria as soon as the hair shafts emerged through the epidermis. Emergence appeared to be slightly delayed in transgenic animals. Furthermore, their fur had a less dense appearance (Fig 1*C* and *D*). In adult mice, transgenic coats always looked rough and less dense as compared with wild-type fur (Fig 1*E* and *F*). The phenotype does not change throughout life. A transgene-specific, non-toxic effect is supported by the compensation of disturbances in and the almost normal appearance of ivl::Igfbp3/ivl::Igf-I double transgenic mice (Fig 1*G* and 1*H*).

To elucidate the reason(s) for the altered coat of transgenic mice, we analyzed the composition of their fur. In wild-type mice, four different hair types occur at characteristic frequencies. About 65%–70% of all hairs are of the zigzag type with three to four sharp bends within the hair shaft. Thirty percent of the pelage is formed by straight hairs called awl. Guard hairs are also straight but significantly longer than awl hairs and occur at a frequency of about 1%–3%. Auchene hairs form a minor fraction with a frequency of about 0.1% and have a single sharp bend. ivl::Igfbp3 transgenic mice possess all major hair types at normal frequencies. A confirmation of the presence of auchene hairs in transgenic mice was impossible because of their very low frequency and of technical limitations of hair isolation that were caused by the severely altered hair structure. Interestingly, the length of transgenic hair shafts of all types is

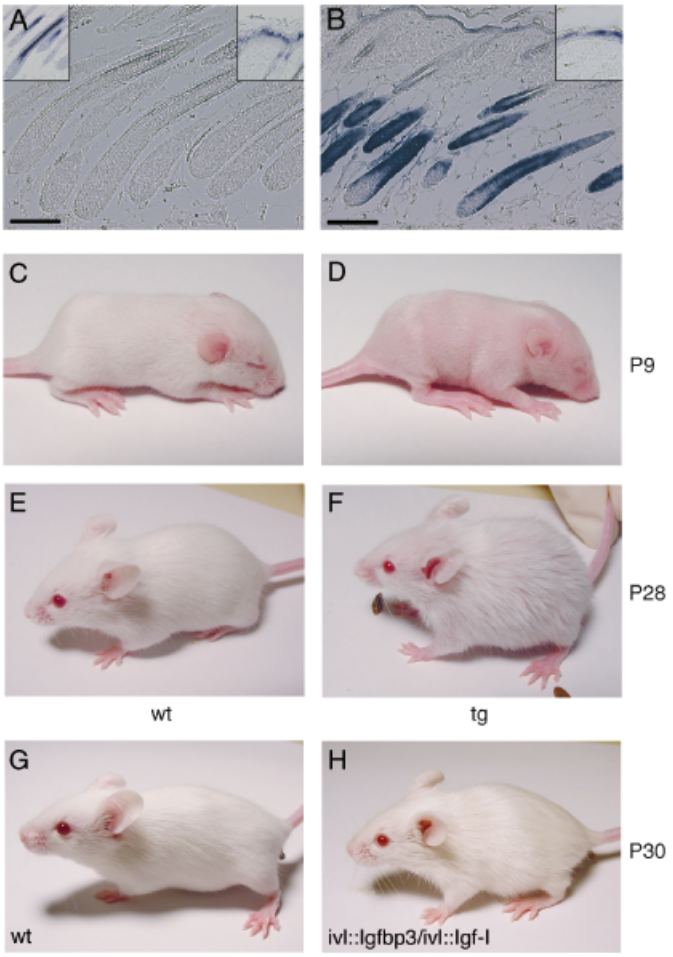

Figure 1
ivl::Igfbp3 transgenic mice develop abnormal coats. (*A*, *B*) Transgenic mice strongly express *Igfbp3* in the mature hair follicle as indicated by non-radioactive *in situ* hybridisations. The insets in (*A*) visualize the expression pattern of the endogenous involucrin gene which is transcriptionally active in the epidermis and the hair medulla. The involucrin promoter fragment drives transgene expression in the epidermis (*inset* in (*B*)) and the medulla as well as the inner root sheath. Expression from the endogenous and the transgenic promoter is much stronger in the hair follicle than in the epidermis. (*C*, *D*) At P9, the skin is still visible in transgenic mice because of a slight delay in the emergence of pelage hairs through the epidermis and to the development of a less dense coat. (*E*, *F*) Adult transgenic mice can still be distinguished from their wild-type littermates by a less dense and rough appearance of the coat. (*G*, *H*) Adult *Igfbp3*/*Igf-I* double transgenic mice are almost indistinguishable from their wild-type littermates. *Scale bars*: 100 μm.

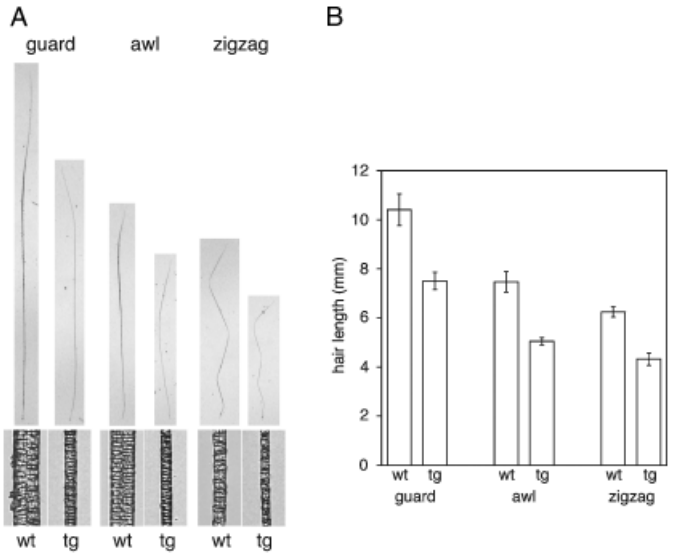

Figure 2
Ectopic expression of *Igfbp3* reduces hair length and thickness. (*A*) A comparison of coat composition demonstrates that transgenic mice still possess all major hair types that are present in wild-type animals (*top*). The number of columns of air spaces is reduced to one in all transgenic hairs as shown by light microscopic analyses (*bottom*). (*B*) Igfbp3-positive hairs are significantly shorter than their wild-type counterparts (all $p < 0.0001$). The mean hair length and the standard deviation are shown ($n = 10$).

significantly reduced to about 70% of their wild-type counterparts ($p < 0.0001$) (Fig 2*A* and *B*). This effect is compensated in ivl::Igfbp3/ivl::Igf-I double transgenic mice (data not shown).

Hair types can be further distinguished with respect to the internal structure of the hair shaft. The medulla forms a regular pattern of air spaces that is visible as a ladder like structure in the light microscope. Whereas wild-type zigzag hairs possess one column of air spaces, guard hairs have two columns. Awl and auchene hairs contain two or more columns of medulla cells. Strikingly, we noted the complete lack of hair shafts with more than one column of medulla cells in ivl::Igfbp3 transgenic mice (Fig 2*A*). Thus, the thickness of hair shafts is clearly reduced. Even transgenic zigzag hairs that contain a single column of medulla cells are thinner than in wild-type mice. Again, these effects are compensated in ivl::Igfbp3/ivl::Igf-I double transgenic mice (data not shown).

Is the less dense appearance of the transgenic pelage a consequence of reduced numbers of hair follicles or is it solely because of the decrease in hair length and thickness? To address this question, we analyzed histological sections of wild-type and transgenic skin that represent anagen. Interestingly, transgenic skin is much thinner than normal which corresponds to the decreased length of hair follicles (Fig 3*A* and *B*). Actually, the overall size of transgenic hair follicles is significantly reduced (bulb thickness: 39.0 ± 4.6 *vs* 51.3 ± 5.1 μm, $p < 0.0001$; follicle length: 0.64 ± 0.08 *vs* 1.09 ± 0.05 mm, $p < 0.0001$). In ivl::Igfbp3/ivl::Igf-I double transgenic mice, follicle size is normal (data not shown). Furthermore, the parallel orientation of hair follicles in wild-type mice seems to be slightly disturbed under the influence of ectopic Igfbp3 expression (Fig 3*B*, *inset*). On the other

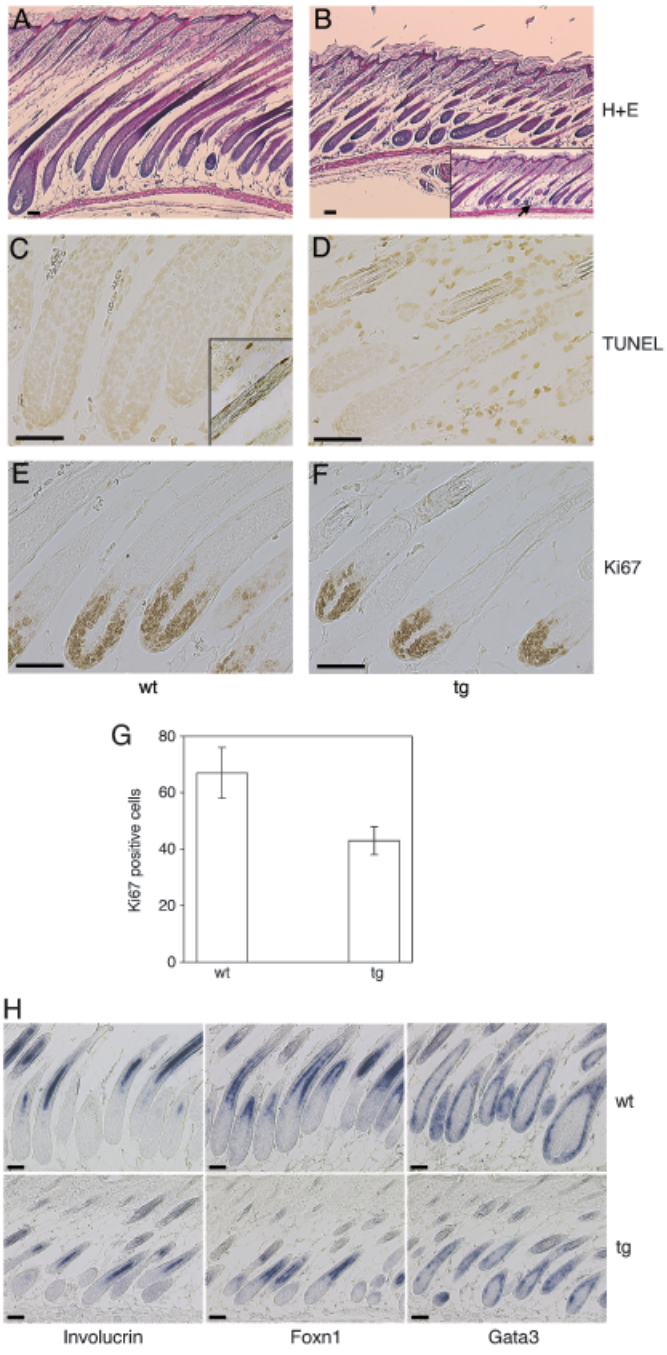

Figure 3
ivl::Igfbp3 transgenic hair follicles are reduced in size and contain fewer proliferating cells. (*A*, *B*) The histology of transgenic skin (P10) was analyzed by hematoxylin and eosin staining of sections. During anagen, transgenic hair follicles are much smaller and shorter than wild-type follicles which causes a significant reduction in skin thickness. The parallel orientation of hair follicles appears to be disturbed in some areas of transgenic skin (arrow, *inset* in *B*). (*C*, *D*) TUNEL assays reveal that transgenic hair follicles do not show excessive apoptosis at P10. Some apoptotic cells are found in the distal inner root sheath (*inset* in (*C*)). (*E*, *F*) Immunohistochemical staining for the proliferation marker Ki67 indicates that the proliferative compartment of hair follicles is markedly reduced in transgenic skin (P10). The ratio of cycling to non-cycling cells in the hair follicle appears to be not affected. (*G*) Quantification of Ki67 positive cells per follicle and section at P10 reveals a 30% reduction in transgenic mice ($p < 0.0001$). The mean number of positive cells and the standard deviation are shown ($n = 15$). (*H*) *In situ* hybridizations reveal that gene expression in distinct hair follicle compartments is not affected in ivl::Igfbp3 transgenic mice at P10. *Scale bars*: 50 μm.

hand, the number of hair follicles is not affected by transgene expression. The density of follicle infundibuli is comparable in wild-type and transgenic skin (Fig 3*A* and *B*).

Is the miniaturization of hair follicles and hair shafts because of excessive cell death or of less cell proliferation? TUNEL staining of wild-type and transgenic skin revealed the absence of any apoptotic cells within the hair shaft forming compartments (Fig 3*C* and *D*). Immunohistochemical staining of proliferating cells demonstrated that their number is reduced in transgenic hair follicles (Fig 3*E* and *F*). Nevertheless, the size reduction of the proliferative compartment is proportional to the overall decrease in hair follicle dimensions (Fig 3*E* and *F*). A quantification of proliferating cells in the hair follicle shows a significant reduction of about 30% in ivl::Igfbp3 transgenic mice as compared with wild-type littermates ($p < 0.0001$) which is in good accordance with the extent of hair and hair follicle miniaturization (Fig 3*G*). The analysis of differentiation marker expression did not offer any indication for an altered differentiation program in any of the follicular compartments. For each domain, at least two different markers were tested (Fig 3*H* and data not shown). Investigation of the expression of various members of the IGF signaling pathway (*Igf-I*, *Igf-II*, *Igf-Ir*, *Igfbp1-2* and *4-6*) also revealed no substantial differences between wild-type and transgenic mice (data not shown).

As in the hair follicle, transgenic *Igfbp3* expression causes hypoplasia in the epidermis, seemingly without any effects on the differentiation program (data not shown). Despite its anti-mitogenic action, *Igfbp3* transgene expression alone is not sufficient to alter the progression through the hair cycle (data not shown). In summary, our data support an antagonistic effect of IGFBP3 and IGF on keratinocyte proliferation in the hair follicle, whereby IGFBP3 most likely contributes to the control of catagen.

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